

学校编码: 10384
学号: 21720091152121

分类号_____ 密级_____
UDC_____

厦 门 大 学

硕士学位论文

海洋放线菌 *Brevibacterium* sp. BS01 杀藻活性物质的分离鉴定及其对塔玛亚历山大藻作用机理的研究

Isolation and identification of algicidal substances produced by *Brevibacterium* sp. BS01 and algicidal mechanism of algicidal compound against *Alexandrium tamarense*

安新丽

指导教师姓名: 郑天凌教授

专 业 名 称: 微生物学

论文提交日期: 2012 年 05 月

论文答辩日期: 2012 年 06 月

学位授予日期: 2012 年 月

答辩委员会主席: _____
评 阅 人: _____

2012年05月

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其它个人或集体已经发表的研究成果,均在文中以适当方式明确标明,并符合法律规范和《厦门大学研究生学术活动规范(试行)》。

另外,该学位论文为(厦门大学应用与环境微生物)课题(组)的研究成果,获得(郑天凌)课题(组)经费或实验室的资助,在(厦门大学应用与环境微生物)实验室完成。(请在以上括号内填写课题或课题组负责人或实验室名称,未有此项声明内容的,可以不作特别声明。)

声明人(签名): 安新丽

2012 年 6 月 7 日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

☐ 1.经厦门大学保密委员会审查核定的保密学位论文，于2013年12月30日解密，解密后适用上述授权。

☒ 2.不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：安新丽

2012年6月7日

目 录

摘 要	1
ABSTRACT	3
第一章 文献综述	6
1 赤潮成因及危害	6
1.1 赤潮的成因	6
1.2 赤潮的危害	8
2 赤潮的控制	10
2.1 赤潮的发生趋势	10
2.2 赤潮的预防策略	12
2.3 赤潮的治理	13
3 杀藻微生物防治赤潮研究现状	14
3.1 海洋微生物治理赤潮	14
3.2 杀藻放线菌的研究进展	15
3.2.1 海洋放线菌及其代谢产物研究进展	15
3.2.2 海洋杀藻放线菌及杀藻活性物质研究进展	16
4 海洋微生物杀藻作用机理	18
4.1 植物化感抑藻的作用机理	18
4.2 微生物抑杀赤潮藻的作用机制研究进展	21
5 本论文研究内容和意义	22
5.1 本论文的研究目的与意义	22
5.2 主要研究内容	23
第二章 海洋放线菌及其杀藻活性物质的分离与鉴定	25
1 材料与方法	25
1.1 实验材料	25
1.1.1 样品采集和实验藻种	25

1.1.2 主要载体	26
1.1.3 主要培养基	26
1.1.4 主要试剂和药品	27
1.1.5 主要仪器	27
1.1.6 主要分析及辅助工具	28
1.2 实验方法	28
1.2.1 杀藻放线菌的分离鉴定	28
1.2.2 杀藻放线菌杀藻活性物质的分离鉴定	28
1.2.3 杀藻化合物鉴定与结构解析	33
1.2.4 杀藻活性物质对塔玛亚历山大藻的抑制作用	34
2 结果	35
2.1 杀藻放线菌分离鉴定	35
2.1.1 杀藻放线菌的分离及菌落形态	35
2.1.2 杀藻放线菌分子鉴定	35
2.2 杀藻放线菌杀藻活性物质鉴定与结构解析	36
2.2.1 杀藻化合物分离纯化	36
2.2.2 杀藻化合物鉴定与结构解析	37
2.3 杀藻活性物质的杀藻效果验证	40
3 讨论	42
第三章 杀藻放线菌杀藻过程追踪	44
1 材料与方法	44
1.1 实验材料	44
1.1.1 实验藻种及菌种	44
1.1.2 实验试剂与仪器	45
1.2 实验方法	46
1.2.1 光学显微镜观察藻细胞形态	46
1.2.2 透射电子显微镜追踪杀藻过程	46
1.2.3 激光共聚焦显微镜观察胞内核酸变化	48
1.2.4 半胱氨酰天冬氨酸特异蛋白酶-3(Caspase-3)酶活性测定	48

2 结果	49
2.1 藻细胞外部形态变化	49
2.2 藻细胞内部形态观察	50
2.3 激光共聚焦观察藻细胞内部核酸变化	52
2.4 Caspase-3 活性测定	53
3 讨论	54
第四章 塔玛亚历山大藻对 BS01 胞外活性物质胁迫的生理响应	55
1 材料与方法	56
1.1 试验材料	56
1.1.1 藻种与放线菌培养条件	56
1.1.2 试验试剂与仪器	56
1.2 试验方法	57
1.2.1 菌株和藻细胞的处理	57
1.2.2 活性氧 (ROS) 含量的测定及荧光显微镜观察	57
1.2.3 ROS 对藻细胞 DNA 的影响	57
1.2.4 藻细胞粗酶液的制备及定量	57
1.2.5 藻细胞超氧化物歧化酶 (SOD) 活性的测定	58
1.2.6 过氧化氢酶 (CAT) 酶活性的测定	58
1.2.7 丙二醛 (MDA) 含量的测定	59
1.2.8 过氧化物酶 (POD) 活性测定	60
1.2.9 杀藻活性物质对藻细胞色素的影响	60
1.2.10 藻细胞叶绿素荧光参数测定	60
2 试验结果	61
2.1 活性氧 (ROS) 测定以及荧光显微镜观察	61
2.2 藻细胞 DNA 变化	62
2.3 藻细胞各种生理指标 (酶活性或含量) 测定结果	63
2.4 杀藻活性物质对藻细胞色素的影响	67
2.5 藻细胞叶绿素荧光参数的测定	68
3 讨论	71

第五章 BS01 胞外活性物质作用下塔玛亚历山大藻基因的表达 ...74

1 材料与方法75

1.1 实验材料75

1.1.1 实验藻种及菌种75

1.1.2 实验试剂与仪器75

1.2 试验方法76

1.2.1 菌株和藻细胞的处理76

1.2.2 藻细胞总 RNA 提取76

1.2.3 总 RNA 样品纯化77

1.2.4 RNA 反转录反应体系77

1.2.5 目的基因片段克隆78

1.2.6 目标靶基因的 real-time PCR 扩增80

2 结果81

2.1 藻细胞 RNA 的提取及反转录81

2.2 目的基因片段克隆结果83

2.3 目标基因 real-timePCR 结果分析84

3 讨论86

第六章 结论与展望89

1 结论89

2 本论文创新点90

3 展望90

参考文献92

附录101

致 谢106

Contents

Abstract (in Chinese)	1
Abstract (in English)	3
Chapter 1 Literature Review	6
1. Cause and hazard of red-tide	6
1.1 Cause of red-tide	6
1.2 Hazard of red-tide	8
2. Prevention and management of red-tide	10
2.1 Developing trend of red-tide	10
2.2 Prevention strategies of red-tide	12
2.3 Control of red-tide	13
3. Advance in the study of microbes mitigating red-tide	14
3.1 Marine microbes controlling red-tide	14
3.2 Advance in controlling HABs using algicidal actinomycetes	15
3.2.1 Marine actinomycetes and active metabolites	15
3.2.2 Algicidal actinomycetes and algicidal materials	16
4. Algicidal mechanisms of marine microbes	18
4.1 Mechanism on the allelopathy of plants	18
4.2 Advance in the study on algicidal mechanisms of marine microbes	21
5. Purpose and significance of this study	22
5.1 Purpose of this study	22
5.2 Significance of this study	23
Chapter 2 Isolation and identification of marine actinomycetes and antialgal substances	25
1. Materials and methods	25
1.1 Experimental materials	25
1.1.1 Seawater sample and dominant alga	25
1.1.2 Main vectors	26

1.1.3 Main media	26
1.1.4 Main reagents and medicines	27
1.1.5 Dominant apparatus	27
1.1.6 Analysis softwares and assistant tools	28
1.2 Experimental methods	28
1.2.1 Isolation and identification of algicidal actinomycete	28
1.2.2 Isolation and purification of algicidal substances	28
1.2.3 Structural analysis of chemical compound	33
1.2.4 Algicidal activity of chemical compound	34
2. Results	35
2.1 Isolation and identification of algicidal actinomycete	35
2.1.1 Colonial morphology and figures of TEM	35
2.1.2 Results of Molecular identification	35
2.2 Identification and structural analysis of chemical compound	36
2.2.1 Isolation and purification of algicidal substance	36
2.2.2 Identification and structural analysis	37
2.3 Validation test of chemical compound	40
3. Discussion	42
Chapter 3 Study on algicidal process of marine actinomycete	44
1. Materials and methods	44
1.1 Experimental materials	44
1.1.1 Alga species and bacterial strains	44
1.1.2 Main reagents and apparatus	45
1.2 Experimental methods	46
1.2.1 Morphological change under optical microscope	46
1.2.2 Algicidal process observed under TEM	46
1.2.3 Dynamic change of nucleic acid in alga cells	48
1.2.4 Enzyme assay of Caspase-3	48
2. Experimental results	49

2.1 External morphology of alga cells	49
2.2 Internal morphology of alga cells	50
2.3 Change of nucleic acid under Laser Scanning Confocal Microscope (LSCM)	52
2.4 Results of Caspase-3 assay	53
3. Discussion	54
Chapter 4 Physiological and biochemical responses of <i>Alexandrium</i> <i>tamarensis</i> treated by supernatant of BS01	55
1. Materials and methods	56
1.1 Experimental materials	56
1.1.1 Alga species and bacterial strains	56
1.1.2 Main reagents and apparatus	56
1.2 Experimental methods	57
1.2.1 Cultivation of alga and strain	57
1.2.2 Determination of ROS and fluorescence observation	57
1.2.3 Damage of DNA induced by ROS	57
1.2.4 Preparation of cellfree extract and quantitative determination	57
1.2.5 Enzyme assay of superoxide dismutase	58
1.2.6 Enzyme assay of catalase	58
1.2.7 Quantitative determination of malondialdehyde	59
1.2.8 Enzyme assay of peroxidase	60
1.2.9 Change of pigments from alga cells	60
1.2.10 Chlorophyll fluorescence parameter of alga cells	60
2. Experimental results	61
2.1 Quantitation of ROS and fluorescence microscope images	61
2.2 DNA damage	60
2.3 Physiological changes(enzyme activity or amount)	63
2.4 Change of pigments amounts	67
2.5 Chlorophyll fluorescence parameter assay	68

3. Discussion	71
Chapter 5 Gene expression of <i>Alexandrium tamarens</i> treated by supernatant of BS01	74
1. Materials and methods	75
1.1 Experimental materials	75
1.1.1 Alga species and bacterial strains	75
1.1.2 Main reagents and apparatus	75
1.2 Experimental methods	76
1.2.1 Cultivation of alga and strain	76
1.2.2 Extraction of total RNA from algae	76
1.2.3 Purification of total RNA	77
1.2.4 Reverse transcription-polymerase chain reaction (RT-PCR)	77
1.2.5 Cloning of target genes	78
1.2.6 Real-time PCR of target genes	80
2. Experimental results	81
2.1 Results of RNA extraction and reverse transcription	81
2.2 Cloning of target genes	83
2.3 Results of real-time PCR	84
3. Discussion	86
Chapter 6 Conclusions, innovations and prospects	89
1. Conclusions	89
2. Innovations	90
3. Prospects	90
References	92
Appendix	101
Acknowledgements	106

摘要

赤潮的控制与防治对于保证海洋环境安全和海洋生态健康是至关重要的。本论文主要研究了海洋杀藻放线菌 BS01 的种属关系；根据 BS01 杀藻活性物质的基本理化性质，分离纯化得到一个具有杀藻活性的化合物；同时探讨了塔玛亚历山大藻在 BS01 胞外分泌物胁迫下生理生化水平的响应；考察藻细胞死亡过程中细胞形态变化；氧化压力下藻细胞光合系统损伤情况；综合运用 real-time PCR 技术研究了藻细胞在杀藻活性物质作用下靶功能基因 *psbA*、*psbD*、*hsp*、*cox* 和 *cob* 转录水平变化。主要研究结果如下：

(1) 分离鉴定得到一株具高效稳定杀藻功能的放线菌，菌株为杆状，属于放线菌目，短杆菌属 *Brevibacterium* sp. (Genbank No. GQ274005)，并将其命名为 BS01。

(2) 从 BS01 的发酵培养物中分离鉴定到一种杀藻化合物 (2-异丁氧基苯基) 胺 ((2-isobutoxyphenyl)amine)，分子量大小为 165。藻细胞在 5 $\mu\text{g/mL}$ ~20 $\mu\text{g/mL}$ 浓度下比较敏感，藻细胞就静沉于瓶底部，光学显微镜下观察失去游动能力，但细胞形态维持完整性，无细胞裂解。

(3) BS01 上清液使藻细胞运动性丧失，质壁分离，藻细胞裂解，叶绿体等细胞器逐渐溶解，内容物溶出，最终整个细胞完全溶解，呈空泡化，推测其经历细胞坏死的过程。在激光共聚焦显微镜下，观察到少数细胞经历细胞程序性死亡的过程，细胞质紧致，细胞核形成类核小体，并呈现趋边化等现象。

(4) 细胞内部活性氧 (ROS) 短时间内显著增加，其体内抗氧化酶体系活性随处理时间和浓度的增加而升高，膜脂质氧化加重，DNA 电泳图像表现出断裂弥散现象。叶绿素 a 和类胡萝卜素降解，12 h 时高浓度处理组 (1.5%) 的光能转换效率明显低于对照，约为对照的 60%。短时间内 (2 h)，藻细胞 ETR 与对照相比皆下降，随着 PAR 的增加，所有处理组的电子传递速率都达到饱和，随处理时间的延长，出现光抑制现象。在 8 h 时，0.5% 处理组的热能散失显著增加，是其他组的将近 2.2 倍。

(5) 荧光定量 PCR 结果表明，*cob* 和 *cox* 在低浓度杀藻活性物质处理下，基因转录水平受到抑制，随着处理浓度的增加，其转录水平也在逐渐的增加；*psbA*

和 *psbD* 分别编码光合系统中两个重要的 D1 和 D2 蛋白, 其荧光定量 PCR 结果表明, 藻细胞光合作用系统受到损伤, 处理初期其转录受到抑制, 后期逐渐增加, 以抵抗外界不良环境所造成的胁迫。*hsp* 是一种应激蛋白, 在本研究中, 其在短时间内没有表现出即时高表达的响应, 而是在系列浓度杀藻活性物质处理下, 其转录水平皆低于对照。

关键词: 杀藻放线菌; 杀藻化合物; 基因转录; 塔玛亚历山大藻

Abstract

Control of red-tide is becoming vital for marine security and ecological recovery. Marine actinomycetes are playing an irreplaceable role on marine biodiversity and stability of marine ecosystem, providing a possible path for controlling red-tide.

In this paper, the typical harmful alga, *Alexandrium tamarense* (ATGD98-006), and algicidal actinomycete *Brevibacterium* sp. BS01 were studied as dominant materials. This study focused on isolation and identification of actinomycetes with the capacity of controlling algae and exploration of algicidal mechanism. Based on physical and chemical characters, a novel algicidal compound was isolated from the culture of BS01. Physiological and biochemical response of algal cells under the stress of supernatant of BS01 was investigated and morphologic changes were observed in the process of cell death. Besides, plant photosynthetic function was explored with the chlorophyll fluorescence kinetics technique. Also, the study included the investigation of expression of target functional genes (*psbA*、*psbD*、*hsp*、*cox* and *cob*) by real-time PCR. There are following main results achieved from these experiments:

(1) A bacterial strain BS01 was isolated and identified from the blooms in Xiamen sea areas and classified as *Brevibacterium* sp. of actinomycete based on microbiological characters and molecular analysis. It was rod-shaped and its Genebank No. was GQ274005.

(2) The co-utilization of silica gel column and gel chromatography mass spectrum as well as nuclear magnetic resonance imaging technique, made (2-isobutoxyphenyl)amine purified from culture of BS01. It was a hydrophobic liquid compound with aniline and a small molecular weight of only 165. *Alexandrium tamarense* was sensitive to 5 $\mu\text{g/mL}$ of (2-isobutoxyphenyl)amine with the loss of motility and sinking at the bottom of flasks, while cells kept intact even under the concentration of 20 $\mu\text{g/mL}$. With the extension of treatment time as well as increasing concentration of supernatant, algicidal effect displayed continuingly obvious.

(3) The death process of algae was monitored with the help of various microscopes, and algal cells were partly clarified as death in the form of necrosis: loss of swimming, occurrence of plasmolysis, lysis of cell wall and membrane, organelles gradually dissolving with efflux of cell contents and whole cell completely dissolving as well as severe vacuolization. Programmed cell death (PCD) was also observed under the laser scanning confocal microscope (LSCM), in which individual algal cells literally destroyed themselves with the production of nucleoid and gradual marginalization.

(4) Reactive oxygen species (ROS) were greatly produced in a short time as a result of attack of active chemical compounds from BS01. Antioxidant enzymes activities were motivated with the adding of concentration of supernatant and extension of treatment time. And ROS could react directly with cellular lipids and DNA, and cause cell oxidative damage, dysfunction, smear of DNA. Oxidative damage to algal cells was thought to be one of main causes of cell death. Meanwhile, the amount of pigments continually decreased and photosynthesis was severely suppressed, resulting in impeding electron transport and ATP formation.

(5) Real-time PCR of *cob* and *cox* showed the similar trend that transcription was impeded when algal cells were treated at low concentration of algicidal compounds, while at high concentration of supernatant from BS01, it showed a continually rising expression level. Genes of *psbA* and *psbD* code proteins D1 and D2 of photosynthetic system, respectively, which are playing vital role in photosynthesis. The results of real-time PCR demonstrated photosynthetic system in *Alexandrium tamatense* was destroyed at the initial stage, and then transcription of the two genes arose with the rising of supernatant concentration, aiming at resisting the adverse environmental impacts. In addition, *hsp* is the gene of coding heat stress protein, which didn't show instantly high expression, but experienced a lower level than control.

Key words: algicidal actinomycetes; algicidal compound; gene transcription; *Alexandrium tamatense*

厦门大学博硕士论文摘要库

Degree papers are in the “[Xiamen University Electronic Theses and Dissertations Database](#)”.

Fulltexts are available in the following ways:

1. If your library is a CALIS member libraries, please log on <http://etd.calis.edu.cn/> and submit requests online, or consult the interlibrary loan department in your library.
2. For users of non-CALIS member libraries, please mail to etd@xmu.edu.cn for delivery details.